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Title: A Convenient Method to Detect Potentially Lethal Heat-Induced Damage to DNA in *Clostridium perfringens*

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Citation: Food Control (2005) 16: 399-404

Number: 7499

A convenient method to detect potentially lethal heat-induced damage to DNA in *Clostridium perfringens* ☆

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Received 4 January 2004; received in revised form 7 April 2004; accepted 8 April 2004

Abstract

A rapid method for determining the lethal effectiveness of heat on a food-borne spore-former based on the conversion of high molecular weight chromosomal DNA to lower molecular weight species was developed. DNA was isolated and purified from spores and vegetative cells of the food-borne pathogen, *Clostridium perfringens*, following exposure to 4, 50, 75, and 100 °C for 15 min. The DNA quality was subsequently analyzed electrophoretically on agarose gels. Spore DNA was most resistant to thermal damage up to 100 °C with minor hydrolysis. Vegetative cell DNA exhibited degradation at 75 °C. A relationship was established between pathogen thermal viability and the electrophoretic stability of DNA. This study describes a convenient method for evaluating heating efficiency based upon electrophoretic DNA stability. These findings may facilitate future testing of pathogen lethality to heat under different menstrum parameters ensuring food safety.

Keywords: DNA; Damage; Heat

1. Introduction

Clostridium perfringens poses a significant threat to the safety of minimally processed foods. The pathogen is capable of surviving stressful conditions including temperatures as high as 100 °C for more than one hour due to the formation of resilient spores (Rhodehamel & Harmon, 1998). Marginal pasteurization temperatures used in the cooking preparations or post heat treatment abuse of ready-to-eat and cook-chill convenience foods may not effectively eliminate the health concerns associated with this microorganism. Previous studies have shown the ability of *C. perfringens* vegetative cells to adapt to nonlethal thermal stress through the synthesis of proteins that provide damage control and enable survival during even higher heating treatment exposures (Heredia, Garcia, Luevanos, Labbe, & Garcia-Alvarado, 1997; Heredia, Labbe, & Garcia-Alvarado, 1998). In attempting to explain the molecular basis for the thermal characteristics of this pathogen the stability of its DNA cannot be underestimated.

Potential targets of heat damage have been implicated with associations to various pathogen viabilities. These include proteins and enzymes, cellular membranes, as well as nucleic acids (Marquis, Sim, & Shin, 1994). Among the implicated targets of heat damage, an argument has been previously made that DNA damage has a greater potential for creating cellular catastrophe as opposed to one of many protein molecules (Setlow, 1992). Recovery from heat damage in *Staphylococcus aureus* involved nucleic acid and not protein synthesis (Sogin & Ordal, 1967). Repair mechanisms must rely on multiple interactions between proteins and DNA. Therefore, as temperatures damage DNA and other vital cellular components, the integrity of DNA cannot be overlooked when assessing the effectiveness of thermal damage on *C. perfringens*.

Examining the integrity of DNA by reduction in molecular weight, via electrophoresis on agarose or acrylamide gels, is a common method examining the genotoxic potential of a xenobiotic or environmental

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stress. The Comet Assay is used in the toxicology field to assess the ability of a xenobiotics ability to induce chromosome damage in eukaryotic cells, and has more recently been used as a detection method for irradiated foods (Cerda, Delincee, Haine, & Rupp, 1997; Tice et al., 2000). Reduction in DNA molecular weight has also been used to evaluate differences in DNA repair capacity of mutant and wild type microorganisms and eukaryotic cells through the generation of endonuclease and exonuclease sensitive sites (Botto et al., 2002; Gulston, Fulford, Jenner, de Lara, & O'Neill, 2002; Minko, Zou, & Loyd, 2002). Gel electrophoresis has also been used to evaluate decreased molecular weight of plasmid DNA following exposure to ionizing radiation and thermal stress (Kim & Thayer, 1996).

Despite the extensive use of electrophoretic mobility of DNA to evaluate genomic stability of microorganisms exposed to external stresses, either chemical or physical, little data exists pertaining to the genomic (DNA) stability of the more common food-borne pathogens following exposure to thermal stresses commonly used in food processing. In order to more fully correlate the effect of thermal processing on genomic stability and cell viability this study examined the quality of DNA isolated from an enterotoxigenic strain, H6, of *C. perfringens* with respect to damage at different temperatures in vegetative cells, spores, and in vitro. Using this simplistic approach, an indication of heating effectiveness on pathogen viability in different food formulations may be compared and pretested in the laboratory, using model systems, by food scientists prior to implementation of the actual food plant processing conditions.

2. Materials and methods

2.1. Microorganism and culture media

Food-borne illness-associated Type A strain, H6 (Hobbs serotype 6) of *C. perfringens*, a very prolific enterotoxin-producing strain was obtained from the Centers for Disease Control (Atlanta, GA). The vegetative cell growth medium consisted of fluid thioglycolate (FTG) medium (Becton Dickinson, Sparks, MD). A modified Duncan and Strong (DS) sporulation medium (Duncan & Strong, 1968) was used for spore preparations. Raffinose was added at 0.4% (w/v) (Labbe & Rey, 1979) in place of starch and caffeine was added following sterilization to a final concentration of 0.5 mM (Juneja, Call, & Miller, 1993). Shahidi Ferguson perfringens (SFP) agar (Beckton Dickinson) consisting of yeast extract, 5.0 g/l; proteose peptone no. 3, 7.5 g/l; pancreatic digest of casein, 7.5 g/l; soytone, 5.0 g/l; ferric ammonium citrate, 1.0 g/l; sodium bisulfite, 1.0 g/l; and agar, 20.0 g/l was used for viability determinations. All chemicals were of the highest quality and obtained from Sigma-Aldrich Chemical Co., Inc. (St. Louis, MO) unless otherwise noted.

2.2. Vegetative cell DNA isolation

Freshly steamed FTG 10 ml culture tubes were inoculated individually with 20 μl each of spore suspension (10 mg/ml) following heat shock at 75 °C for 20 min. The FTG tubes were incubated at 37 °C with spectral absorbance readings taken every 30 min to an OD of 1.0 at 600 nm. The vegetative cells were centrifuged and washed (3×) with 0.15 M NaCl prior to resuspension in sterile distilled water (dH_2O) in sterile microfuge tubes. The cells were then treated in duplicate by immersion in 4, 50, 75, and 100 °C water baths for 15 min, respectively. The cells were pelleted and the DNA isolated (Sakakibara, Tanooka, & Terano, 1970).

2.3. Isolation of free spore DNA

Freshly steamed tubes of FTG medium were inoculated with *C. perfringens* H6 spores following a heat shock of 75 °C for 20 min. The tubes were incubated at 37 °C overnight. One ml of the overnight culture was then inoculated into 10 ml of freshly steamed DS sporulation medium. Incubation was continued at 37 °C until 90% sporulation, determined microscopically, was obtained. Spores free from vegetative cells were obtained using the method of Durban, Durban, and Grecz (1974) as modified from Grecz, Anellis, and Schneider (1962). Spores were resuspended at 10^8 spores/ml in sterile dH_2O in sterile microfuge tubes. These were then treated in duplicate by immersion in 4, 50, 75, and 100 °C water baths for 15 min, respectively. Spore spheroplasts were prepared (Durban et al., 1974) and DNA isolated as previously described (Sakakibara et al., 1970).

2.4. Cell-free DNA treatments

One microgram quantities of vegetative cell DNA isolated from 4 °C treated cells were resuspended in sterile dH_2O in sterile microfuge tubes as was commercially obtained *C. perfringens* DNA (Sigma-Aldrich Chemical Co., Inc., St. Louis, MO). The DNA containing tubes were treated in duplicate by immersion in 4, 50, 75, and 100 °C water baths for 15 min, respectively. Following heat treatments, and respective isolating methods, gel-loading buffer was added. The DNA samples were loaded onto standard 0.7% agarose gels (FMC BioProducts, Rockland, ME) and DNA quality was analyzed following electrophoresis (Sambrook, Fritsch, & Maniatis, 1989).

2.5. Enumeration of survivors

An overnight mixed culture (vegetative cells and spores) of *C. perfringens* H6 cells grown in FTG medium at 37 °C to a concentration of 5.0×10^6 viable cells/ml with 1.3×10^5 spores/ml or 2.56% spores was chosen to validate heat treatment effects as homogeneous cell or spore populations were considered improbable in a temperature compromised or contaminated food scenario. Survivors (from spores and vegetative cells) from the various treatment conditions (0, 15, 30, 60, and 120 min at 4, 50, 75, and 100 °C) were serially diluted in 0.1% (w/v) peptone–water and plated on SFP medium to obtain viable CFU/ml. After 30 min an SFP overlay was added to the plates and incubated at 37 °C for 18 h under anaerobic conditions (10% CO_2, 5% H_2, 85% N_2) using a Bactron IV anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR), followed by enumeration of surviving cells.

3. Results

3.1. Thermal stability of C. perfringens DNA in vitro

Microgram quantities of *C. perfringens* genomic DNA were isolated, resuspended in sterile dH_2O, heat-treated, and compared following agarose gel electrophoresis with commercially obtained *C. perfringens* type XII DNA (Fig. 1A). The cell-free *C. perfringens* DNA experienced a small reduction in size following 75 °C for 15 min (Fig. 1A, lane 4). Complete hydrolysis was evident following 15 min at 100 °C (Fig. 1A, lane 5). Commercially obtained *C. perfringens* DNA exhibited similar trends, although the genomic DNA was initially sheared, giving a progressively lower range of DNA smears in the lanes corresponding to increasing temperature exposures (Fig. 1A, lanes 6–9). The DNA preparations documented in this study also resulted in large quantities of RNA shown to be smaller than 1 kb in size (Fig. 1A, lanes 2–5). Removal of the low molecular weight RNA was confirmed following digestion with RNase.

3.2. Thermal stability of vegetative cell and spore DNA in vivo

The *C. perfringens* DNA exposed to heat treatments while inside vegetative cells appeared to degrade following the 75 °C exposure (Fig. 1B, lane 4). Likewise, complete hydrolysis was evident following 15 min at 100 °C (Fig. 1B, lane 5). The DNA exposed to heat treatments within spores was resistant to thermal effects beyond 75 °C for 15 min (Fig. 1B, lane 9). Spore DNA hydrolysis is evident following 100 °C for 15 min (Fig. 1B, lane 10). Less DNA was isolated from 10^8 spores as compared with vegetative cell preparations. RNA was present at 2 kb in size (Fig. 1B, lanes 7–10). Removal of the RNA was confirmed following digestion with RNase.

3.3. C. perfringens culture thermal viability

A representative vegetative cell and spore mixed culture of *C. perfringens* H6 was used to depict the effects of temperature on the survival of this food-borne pathogen. The cells were then exposed to 4, 50, 75, or

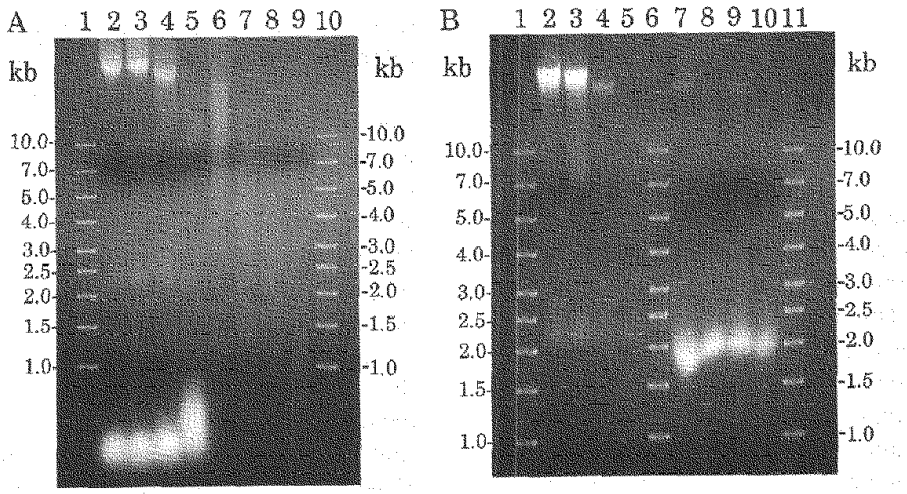

Fig. 1. (A) Microgram quantities of purified *C. perfringens* strain H6 cell-free in vitro vegetative (V) and commercially-obtained (C) genomic DNA exposed to varying temperatures for 15 min followed by electrophoretic migration through 0.7% (W/V) agarose gel. Lanes: 1, DNA standard ladder; 2, V DNA/4 °C; 3, V DNA/50 °C; 4, V DNA/75 °C; 5, V DNA/100 °C; 6, C DNA/4 °C; 7, C DNA/50 °C; 8, C DNA/75 °C; 9, C DNA/100 °C; and 10, DNA standard ladder. (B) Microgram quantities of purified *C. perfringens* strain H6 vegetative (V) and spore (S) genomic DNA in vivo exposed to varying temperatures for 15 min followed by electrophoretic migration through 0.7% (W/V) agarose gel. Lanes: 1, DNA standard ladder; 2, V DNA/4 °C; 3, V DNA/50 °C; 4, V DNA/75 °C; 5, V DNA/100 °C; 6, DNA standard ladder; 7, S DNA/4 °C; 8, S DNA/50 °C; 9, S DNA/75 °C; 10, S DNA/100 °C; and 11, DNA standard ladder.

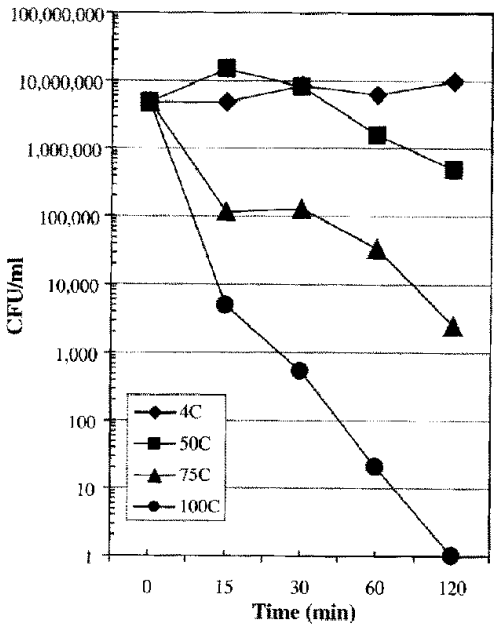

Fig. 2. Temperature effects on *C. perfringens* strain H6 culture viability. Surviving culture cells (5.0×10^6 vegetative cells/ml and 1.3×10^5 spores/ml initial) measured as CFU/ML following a 0–120 min exposure at 4, 50, 75, and 100 °C. Results depicted are the average of duplicate experiments.

100 °C temperatures for up to 120 min in duration (Fig. 2). Culture viability appeared stable at 4C (Fig. 2). Following 60 min at 50 °C, viable cells decreased approximately one logarithm base 10 (Fig. 2). For the 75 and 100 °C treatments, a 2 and 3 log decrease in cell viability was exhibited after 15 min, respectively (Fig. 2). All of the survivors at 75 and 100 °C could be represented by the viable spore population. A small number of *C. perfringens* spores were still viable following 100 °C for 60 min, but there were no survivors following 120 min (Fig. 2).

4. Discussion

The integrity and stability of the DNA isolated from *C. perfringens* enterotoxigenic strain H6 gives an indication of this pathogen's ability to survive different heating regimens. This is in agreement with the correlation between the qualitative measurement of DNA degradation and cell viability traditionally made using other systems (Cerda et al., 1997; Kim & Thayer, 1996; Tice et al., 2000), and draws attention to the fact that this is the first attempt at evaluating the stability of DNA with respect to both *C. perfringens* spores and vegetative cells.

DNA melting has been shown previously to occur at temperatures above 90 °C (Mackey, Miles, Parsons, & Seymour, 1991; Teixeira, Castro, Mohacsi-Farkas, & Kirby, 1997). In various microorganisms cellular death below such high temperatures was believed to be attributable to other equally important cellular components such as degradation of ribosomes (Sogin & Ordal, 1967), ribonucleic acids (Russell & Harries, 1968), or proteins (Belliveau, Beaman, Pankratz, & Gerhardt, 1992). To this end, a single critical component has not been identified. It is possible that different temperatures target different crucial components and only those molecular constituents in high concentrations exhibit any noticeable effects.

Even mild heat is known to play a key role in DNA damage and hydrolysis. Purine bases were first shown to be liberated from DNA at elevated temperatures (80–95 °C) followed by depyrimidation of cytosine and thymine bases at approximately 5% the rate of depurination (Lindahl & Karlstrom, 1973; Lindahl & Nyberg, 1972). It was found that apurinic sites led to greater than 95% chain breakage over control DNA (Lindahl & Anderson, 1972). The chain breakage was considered a significant mechanism of heat induced lethality in bacterial spores (Grecz & Bruszer, 1981). More recently it has been found that covalently closed circular plasmid DNA can initially withstand denaturation at temperatures of 95–107 °C, but subsequent depurination events eventually lead to DNA strand breakage followed by denaturation (Masters, Miles, & Mackey, 1998).

We have shown that DNA damage for *C. perfringens* vegetative cells can be easily demonstrated by electrophoretic characterizations on agarose gels well below the temperatures necessary for DNA melting (Fig. 1A and B). DNA hydrolysis was evident after only 15 min at 75 °C in vegetative cells. At this temperature, corresponding cell cultures also decreased in viability (Fig. 2). Previously, sporulation mutations had been found to be induced by heat and the spore DNA was presumed to be different from vegetative DNA in some properties (Northrop & Slepecky, 1967). The differences in thermal stabilities of vegetative cells and spores appear to coincide with the electrophoretic appearance of DNA isolated from the different cell types used in this study. The *C. perfringens* DNA isolated from within spores was shown to be stable to almost 100 °C (Fig. 1B). While the DNA from within vegetative cells was only stable to 50 °C (Fig. 1B), purified DNA free from cells was found to be similarly heat labile (Fig. 1A).

Spores generally have heat resistance mechanisms that extend heat tolerances above vegetative cells. DNA repair mechanisms have been shown to have a significant influence on heat resistance (Hanlin, Lombardi, & Slepecky, 1985). The heat resistance of spores is believed to be attributed in part to restricting the mobility of heat labile components of the spore core such as proteins and DNA (Gombas, 1983). Spore DNA is further protected by small acid soluble proteins (SASPs) that bind tightly and specifically to the A form of DNA reducing the rate of depurination in vitro by at least 20-fold (Fairhead,

Setlow, & Setlow, 1993; Lindahl, 1993; Setlow, 1994; Setlow, 1995). Additional evidence in support of the role of DNA in thermoresistance was found in an observed correlation between spore DNA content and elevated heat resistance (Belliveau, Beaman, & Gerhardt, 1990). Dipicolinic acid (DPA) or calcium-DPA complexes with DNA were also correlated with spore heat resistance (Lindsay & Murrell, 1985). Whatever the DNA protective mechanism involved, DNA stability is vital to thermoresistance in a microorganism.

The generally accepted method of determining thermoresistance involves the cellular survival calculation of *D*-values based on the base 10 logarithmic reduction times of initial cell populations in response to specific heat treatment conditions. Typically, results are not obtained in less than 48 h due to cellular growth and extensive data calculations. This study indicates that an 8 h evaluation of heating menstrums on electrophoretic DNA stability as an initial measure of cell lethality prior to expensive and time consuming *D*-value determination, may be of use in model food processing systems. These techniques used to evaluate chromosomal DNA integrity require minimal training. Equipment costs for electrophoresis equipment are generally low and would be offset by the ability of processors to quickly test the effectiveness of conditions to improve microbial safety for specific products. To summarize, this method can be of use to Food Scientists in pre-trials of new formulations with respect to evaluating processing guidelines for the control of food-borne pathogens.

Acknowledgement

We wish to thank Ms. Kenyetta Chaney for provided technical assistance.

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